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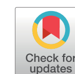
Tubulin-based polymorphism genome profiling: a novel method for animal species authentication in meat and poultry



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ABSTRACT

We present a new method for the authentication of the biological identity of raw meat and processed meat products that is based on length polymorphism found in the introns of the members of the animal beta-tubulin gene family. The method, denominated aTBP for animal Tubulin-Based-Polymorphism, is shown to be capable of assigning an exclusive genomic fingerprinting to ten different animal species, eight of which are largely consumed as food products. Besides an exclusive DNA profiling, each species is characterized by the presence of specific diagnostic fragments, that assist their selective recognition in admixtures and products sold in the market. The aTBP method is also shown to be effective in both DNA/DNA and weight/weight mixtures where the presence of the low abundance species can be detected at the level of 0.5% and 1% respectively. Detection by aTBP genome profiling is also obtained from either frozen/thawed or cooked samples. The composition of 25 market products made by meat was also assessed with respect to what declared in the label. The results are discussed with reference to biosurveillance and disclosure of frauds and contaminations in comparison with other DNA-based diagnostic methods currently used.

1. Introduction

Food biosurveillance is important for recognition of those market products where the genetic identity of the components may be unclear and high is the concern about substitutions, adulterations, frauds, health risks and possible violations of ethical and religious principles. Paradigmatic, in this respect, has been the horse meat case emerged in Europe in 2013, although a major scandal in meat substitution was actually recorded much earlier, in 1981 in Australia, when any sort of animal species was found in pet food, including game killed in the field, without any concern for hygiene and safety issues. Because of this, considerable quantities of pet food were illegally diverted into the human food chain (Grabosky, 1989). Several years later, the percentage amount of substitution for common species in meat and derived-meat products was still ranging from 20% to 70%, according to many reports that referred to different countries (Ayaz, Ayaz, & Erol, 2006; Cawthorn DM et al., 2013; Di Pinto et al., 2015; Okuma & Hellberg, 2015; Quinto, Tinoco, & Hellberg, 2016; Kane & Hellberg, 2016). According to a recent report of the European Administrative Assistance and Cooperation (AAC) system, alleged violations in the meat sector are by far the most common compared to other food sectors, and the vast majority relates to mislabeling composition (EU ACC report, 2016). In fact, meat is easily susceptible to fraudulent substitutions with less valuable meat or even domestic animals. Without disregarding the important contribute of other diagnostic techniques such as ELISA (Kang'ethe, Jones, & Patterson, 1982), HPLC (Andrasko & Rosén, 1994), NIR (Weeranantanaphan, Downey, Allen, & Sun, 2011), FT-NIR (Alamprese, Amigo, Casiraghi, & Engelsen, 2016), FT-IR (Hu, Zou, Huang, & Lu, 2017), GC (Czesny, Dabrowski, Christensen, Van Eenennaam, & Doroshov, 2000), NMR (Ralli et al., 2018), HPLC-MS/MS (von Bargen, Brockmeyer, & Humpf, 2014) and others (reviewed in Sentandreu & Sentandreu, 2014), it is becoming more and more evident that molecular, DNA-based tools represent much of the present and of the future of the biosurvellaince field as also emerged from the European directives on food labeling (EU regulation, 25AD No 1169/2011). This is because DNA is normally more resistant to industrial processes compared to other biomolecules. Moreover, DNA-based methods are generally more specific, more sensitive and less expensive than other techniques (Bohme, Calo-Mata, Barros-Velasquez, & Ortea, 2019). Among them, RT-PCR is one of the most frequently used also for the authentication of meat products, especially when it is multiplexed, so that many target genes, diagnostic of different, selected species, can be assayed in one reaction (Meira et al., 2017; Xu et al., 2018; Wang, Liu, Zhang, Zhou, & Liu, 2019). RT-PCR doesn't depend on electrophoresis or DNA sequencing and can also provide quantitative data. HRM, for High Resolution Melting, can also discriminate among animal species because of differences of the melting profiles of the

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fragments that are previously amplified by PCR. HRM-based screening of meat samples looking for adulteration may lead to the identification of the different species present in a mixture and of their relative proportion (Lopez-Oceja, Nunez, Baeta, Gamarra, & de Pancorbo, 2017). Also ddPCR (digital drop PCR) has been used to identify and quantify species in meat and meat products (Floren, Wiedemann, Brenig, Schutz, & Beck, 2015). While species is the taxonomic level of highest importance when assessing the biological origin of food products, some attempts, based on multiple single nucleotide polymorphisms, have also been made to distinguish different breeds and subpopulations (Xing et al., 2017). However, RT-PCR, HRM and ddPCR-based methods are all specifically designed to target the species of interest. They do not uncover the presence of undeclared or unexpected species. Additional DNA-based diagnostic procedures combines PCR amplification with either electrophoresis (Rahman et al., 2015) or traditional DNA sequencing of specific target regions, thus resulting in the assignment of a specific DNA barcode (Luo et al., 2011; Lo & Shaw, 2018). The former approach is limited by the number of amplicons that may be produced since a high number of amplicons of similar sizes may reduce their resolution by electrophoresis while the latter may encounter problems with concurrent multiple species identification and the detection of undeclared species, as reported by several laboratories (Hellberg & Morrisey, 2011; Galimberti et al., 2013; Morello, Braglia, Gavazzi, Gianì, & Breviario, 2019). More recently the application of NGS technologies for the determination of meat adulteration has been reported (Ripp et al., 2014; Giusti, Armani, & Sotelo, 2017) but some limits related to the selection and length of the target sequence, the construction or the availability of the library of reference, the amount of the bionformatic work and the real cost of the analysis including the cost and the maintenance of the equipment, makes this approach still incomplete and unaffordable by numerous, small scale laboratories. The TBP (Tubulin Based Polymorphism) method, originally developed for plants (Gavazzi et al., 2016; Braglia et al., 2016, 2018), may offer a valid, simple, competitive and rather inexpensive alternative, also applicable to the detection of undeclared or unexpected animal species. It is based on the intron length polymorphism that is typically found in the genes encoding for animal beta-tubulin, a protein that is a key constituent of the cellular microtubules. The key role played by microtubules in cell division reflects in the conservative exon-intron organization of the beta-tubulin genes sketched at the top of Fig. 1. A total of three introns may be present in any gene encoding for vertebrate beta-tubulin, there are two in plants, and their positioning within the coding sequence is strictly conserved. This allows simple PCR-mediated amplification of any of the intervening intron sequences, once a pair of generalist primers, capable of annealing to the exon boundaries of any vertebrate species, is designed. Because each intron has its own length (and nucleotide sequence composition) and different species may contain a different number of beta-tubulin genes, hence a different number of introns, PCR amplification results in a multiple fragments profile, capable, in principle, to characterize any vertebrate species (Fig. 1). This newly devised version of the TBP method has been named aTBP for animal Tubulin-Based-Polymorphism. As reported here, the aTBP method is simple, largely applicable to single components, for purity assessment, and to mixtures for identification of ingredients at 1% w/w, a pragmatic and largely accepted threshold, by current regulations (Food Standards Agency, July 2013). aTBP is not dependent on DNA sequencing, assist the detection of unanticipated food components and can be conveniently used as a practical screening methodology. Food adulterations of relevance for this paper are those concerning animal species substitution or dilution/mixing with other species.

2. Materials and methods

2.1. Experimental samples

Edible raw meat samples of beef, sheep, pork, goat, horse, chicken, rabbit and turkey were either purchased at the supermarket or provided by the Consortium of the producers of Parco del Ticino (Sig. Sandro Passerini). Animal breed specific DNA for beef, sheep, goat and pork was kindly provided by Dr. Stefania Chessa, Turin University, Italy. Mouse DNA was a gift of Dr. Filippo Turrini, San Raffaele Hospital (HSR), Milan, Italy. Human genomic DNA was from the authors of this paper. A more detailed list of the material used in this study is made available as supplementary information (Supplementary Table 1). Commercial products were purchased from local supermarkets while stock cubes, solid cube made from meat or vegetables, commonly used to prepare soups, were homemade as follows: 200 gr of minced beef, 200 gr of mixed vegetables in pieces, 200 g of salt, 20 g of wine and different plant aromas, all cooked with the Vorwerk Thermomix (Bimby).

2.2. DNA extraction

200 mg of each edible meat sample or 2 gr from meat mixtures (described in sections 2.4) were ground with mortar and pestle in the presence of liquid nitrogen. Genomic DNA was extracted using *DNeasy^R Mericon^{TM} Food Kit* (Qiagen) according with the manufacturer's instructions. DNA quality and quantity were assessed by nanodrop-2000C spectrophotometer (Thermo Fisher Scientific). DNA integrity was evaluated by 0,8% agarose gel analysis and *Atlas Clear Sight DNA* stain (1 μg ml^{-1}), using *Gene Ruler^{TM}1 kb plus ladder* (Thermo Scientific) for reference. As reported below, the number of independent DNA extractions was two for DNA mixtures and three for meat mixtures. Negative controls were made by using the same reagents and applying the same protocols in the absence of animal DNA or tissue.

2.3. DNA mixtures

DNA extracted from horse and beef meat samples were mixed in a 1:1 quantitative ratio whereas 0.5%, 1%, 3% and 5% increasing amount of either pork or horse DNA were spiked into DNA extracted from chicken or beef, respectively. Every DNA mix was made in duplicate.

2.4. Meat mixtures

Grinding was performed as follows: 200 g each of beef, chicken and pork (M1) or beef, chicken and horse meat (M2) were shredded by hand with a knife and then ground, with a different kitchen robot (Broun, multi quick 3) to avoid accidental contamination. A mixture made up by 116,7 g each of beef, chicken and pork (M1) or beef, chicken and horse (M2) was then mixed to prepare two mixtures at a 1:1:1 w/w ratio for a total 350 g mixed sample. In the adulteration assays, 99, 97 and 95 g of M1 and M2 were added with 1, 3 and 5 g of horse and pork respectively, previously ground as already described, to obtain a final w/w percentage of 1%, 3% and 5%. The left over amounting to 50 g for each M1 and M2 mixture was used as a negative control. Each mixture, adulterated or no, was made in triplicate. Assays on defrosted or cooked meat samples, were performed on 2 g of the M1 mixture or M1 spiked with increasing amount of horse meat. Samples were either thawed after 48 h of freezing at −20 °C, or dispersed in 1 ml of distilled water and cooked in a microwave oven for 20 s at 465 W, as reported by Alamprese et al. (2016).

2.5. TBP amplification and capillary electrophoresis (CE-TBP)

TBP amplification of beta-tubulin intron III was performed using degenerated primers aFex3 (5′- GAYYTDGARCCNGGNACNATGG-3′) and aRex3.2 (GTRTAGTRVCCYTTNGCCCAGTTG) designed in the region straddling intron III and boundary exons III and IV, based on the alignment of 29 cDNA sequences of vertebrate beta tubulin deposited in the NCBI Genebank with the following accession numbers: *Bos taurus*: NM_001144100.1, NM_001003900.1, NM_001144100.1; *Ovis aries*: XM_027969782.1, XM_027958682.1; *Sus scrofa*: NM_001113696,

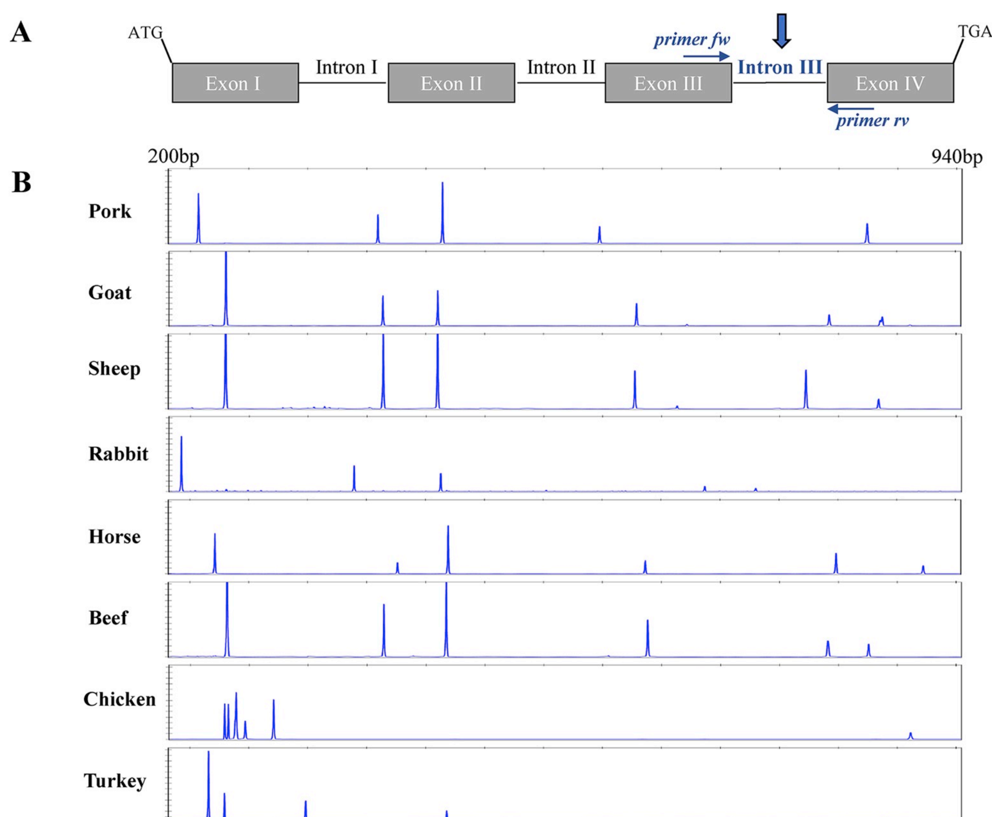


Fig. 1. Genomic profiling by aTBP. A: the genomic organization of a generic animal beta-tubulin locus is shown. Intron III, the source of DNA length polymorphism, is amplified by the use of an all-purpose primer pair, applicable to animal species. B: CE-TBP electropherograms of eight different animal species, commonly present in food. Sizes of the amplicons spans from 200 bp to 940 bp while peaks height goes from 100 to 20.000 RFU values.

NM_001243434.1; *Gallus gallus*: NM_001004400.2, NM_001031598.1, NM_001080860; *Equus caballus*: XM_001491178.3, M_001490328.2, XM_001914774.2; *Meleagris gallopavo*: XM_003212121; *Capra hircus*: XM_013973992.2; *Oryctolagus cuniculus*: XM_002720955.3, XM_002714359.2, XM_008252902.1; *Danio rerio*: BC056533.1; *Oreochromis niloticus*: XM_003452414.5, XM_003454595.5, XM_005458819.4; *Salmo salar*: NM_001139793.1, XM_014175699; *Ictalurus punctatus*: XM_017465362.1, XM_017481202.1; *Gadus morua*: AF102890.1; *Homo sapiens*: NM_006088.6; *Mus musculus*: NM_011655.5. aTBP primer sequence is protected by the European patent n. 3011049. For TBP amplification followed by capillary electrophoresis, forward primer aFex3, was 6-FAM-labeled at its 5′-end.

The following amount of different material was used as template for each aTBP amplification: 50 ng of genomic DNA from raw meat samples, 150 ng from raw food samples and mix DNA (1:1 ratio), 300 ng from 1%, 3%, 5% spiked meat mix and from food that was subjected to physical treatments. Negative controls, with no DNA, were always included. Each reaction was performed in 30 µl of final volume with 1x Taq Polymerase Master Mix (2x, 2 mM MgCl2; VWR). Following the initial denaturation step at 94 °C for 3 min, the PCR reaction continues with the following touchdown thermal profile: 14 cycles of 30 s at 94 °C, 45 s at 67 °C, (decreasing by 0.7 °C every cycle), 2 min at 72 °C; 25 cycles of 30 s at 98 °C, 45 s at 57 °C, 2 min at 72 °C; final extension at 72 °C for 30 min.

PCR reactions were first loaded on 2% agarose gel using the 1 Kb plus marker as reference to verify the intensity of the amplification signal so to proceed with the appropriate dilutions to be used for the CE-mediated, amplicon resolution analysis. CE-TBP was performed on a 3500 Genetic Analyser (Thermo Fischer Scientific) as described by Gavazzi et al. (2016).

The data referring to fragment sizes and peak intensity (RFU) were collected using the Data Collection Software v. 3.1 (Thermo Fisher Scientific) and then analyzed by the GeneMapper Software v. 5.0 tool (Thermo Fisher Scientific). Data analysis was made by simple comparison of the numerical output of the AB 3500, conveniently converted in an Excel spreadsheet which allows to associate each animal species with its specific amplicons profile. All electrophoretic runs were repeated at least twice for each independent experiment to confirm the profiles. The whole procedure, from amplification to DNA profiling, applied to eight samples, in correspondence to the eight capillaries of the 3500 Genetic Analyser, takes one day of work followed by an overnight electrophoretic run.

2.6. Gene bank sequence analysis

Genomic sequences encoding animal beta tubulins were retrieved from the Ensembl Genome browser (https://www.ensembl.org/index.html) either by the "search" function, using "tubulin" as a keyword, or by BLAST search, using a rice beta tubulin amino acid sequence as reference. Reported intron positions were verified by alignment with a reference tubulin cDNA, and adjusted if needed, to infer the correct intron length reported in Table 1. When not present in Ensembl, the gene name was putatively assigned based on intron length similarity with related species.

3. Results

3.1. The aTBP method: application to different animal species

The aTBP version of the original plant method described in this paper has been developed on intron III and relative exon boundaries, because, after several preliminary attempts, it was found to be the most reliable and consistent source for detecting DNA polymorphisms in animal species (Fig. 1). As reported in Fig. 1 and Table 1, the application of the aTBP method to a total of ten different animal species, eight of which commonly consumed as meat by humans (Fig. 1) and two possible contaminating species (Table 1), resulted in the production of clearly distinguishable, species-specific genomic profiles. The aTBP electropherograms are characterized by the presence of a different number of amplification fragments, (peaks in CE), each having different

Table 1
Comparison between the expected size, calculated from WSG data available in Ensembl, and the size of the amplicons generated by aTBP.

			Amplicon Size (bp)								
			TUBB	TUBB1	TUBB2A	TUBB2B	TUBB3	TUBB4A	TUBB4B	TUBB6	TUBB8
MAMMALS	*Sus scrofa* (pork)	Ensembl	395	230		454	864	3086	606	3578	
		CE-TBP	**395**	**229**		**456**	**851**	*	**602**	*	
	Capra hircus (goat)	Ensembl	400	255	824	451	873	2688	639	5384	
		CE-TBP	400	253	**818**	451	865/867	*	**637**	*	
	Ovis aries (sheep)	Ensembl	400	255	871		800	2687		partial	
		CE-TBP	400	253	**855/862**	451	**795/797**	*	**635**		
	Oryctolagus cuniculus (rabbit)	Ensembl	374	213	706	455	757	2686		14442	
		CE-TBP	**373**	**211**	**700**	**454**	**748**	*		*	
	Equus caballus (horse)	Ensembl	partial	245	828	415	916	649	2667	12500	
		CE-TBP	462	243	**825**	**415**	**907**	647	*	*	
	Bos taurus (beef)	Ensembl	401	256	824	459	861	3505	650	4797	
		CE-TBP	401	255	**816**	459	**853**	*	646		
	Mus musculus (mouse)	Ensembl	243^	223	769	460	1676	4364	685	8373	
		CE-TBP	243	**222**	**767**	461	*	*	**685**	*	
	Homo sapiens (man)	Ensembl	431	224	821	467	1270	5185	594	14133	620
		CE-TBP	**431**	**224/228**	**823**	**468**	*	*	**590**	*	**612/618**
BIRDS	*Gallus gallus* (chicken)	Ensembl	907	257	276	302			516	4578	
		CE-TBP	**896**	253/255	**263/271**	**299/301**			not found	*	
	Meleagris gallopavo (turkey)	Ensembl		238		331				4229	
		CE-TBP	868	**238**	253	**328/329**			460	*	

Squared numbers indicate those TBP amplicons whose corresponding genomic sequence was not present in Ensembl. Gene names were assigned based on sequence length similarity with more related species. Double numbers indicate allele variants.
^ annotated as an unknown Tub5 in Ensembl; * = amplicon length exceeding CE resolution limit. Numbers in bold indicate species-specific, diagnostic amplicons, with a 2 bp tolerance; https://www.ensembl.org/index.html.

length, different electrophoretic mobility (expressed in bp) and different Relative Fluorescence Unit (RFU) values, these latter generally higher for the shorter fragments (Fig. 1; mouse and human not shown). The reproducibility and specificity of the aTBP genomic profiles have been assessed by multiple independent determinations, performed on each of the ten animal species, and by running animal/plant cross amplification reactions to verify that the aTBP primers were ineffective on plant DNA and vice versa (data not shown). Also, the identity of some of the fragments amplified by the aTBP method was ascertained by DNA sequencing that allowed the recognition of the exon boundaries sequences, and typical donor/acceptor splice sites, unequivocally attributable to beta-tubulin genes, as also verified by comparing the sequences with those present in the corresponding genome databases. To this specific regard, a remarkable consistency was found comparing the sizes of the aTBP amplified fragments, resolved by capillary electrophoresis, with those of corresponding DNA sequence data retrieved from the Ensembl genome browser for vertebrates corresponding to all the different animal species investigated, as verifiable in Table 1. Gene names are those reported in the database, with reference to human tubulins. Consistency between aTBP and DNA sequence data was found up to the 900 bp long fragments, while larger amplicons, like those of *TUBB6* and *TUBB4*, could not be detected since they exceed the limit of resolution of the CE technique that is 1.2 kb, in our experimental conditions (asterisks in Table 1). Below this limit, all but one of the predicted fragments (chicken *TUBB4B*, 516 bp) were amplified by aTBP, while all but five TBP amplicons (three from turkey and two from sheep) could be attributed to the respective locus (squared numbers in Table 1). Assignment of such aTBP amplicons to specific tubulin genes was done by length similarity with more related species. The five missing intron sequences are due to incomplete gene sequences retrieved from the genome assembly. The few length differences reported in Table 1 may be attributed to minor inaccuracies either in peak size attribution by the capillary electrophoresis software or to imperfect genomic DNA sequences and, in some case, to the occurrence of short InDel polymorphisms not yet annotated in the data base. In fact, since the TBP marker is codominant, amplicons of similar sizes found in different samples of the same species, likely corresponding to different alleles, could associate to the same locus identified in the Ensembl database (double numbers in Table 1). A similar finding, that is an unrecorded beta-tubulin allele, was also obtained by the TBP genotyping of grape (Gavazzi et al., 2016). This may also be the case for chicken loci *TUBB1*, *TUBB2A* and *TUBB2B*, showing alternative alleles, possibly associated to different breeds. In fact, they contribute to define four different aTBP profiles, one of which was readily recognizable in some market products like hamburger, kebab and canned grilled chicken (Supplementary Fig. 1). Scoring all the detectable fragments reported in Table 1, at least two species-specific diagnostic peaks, corresponding to amplicons of distinctive sizes, with the allowance of a 2bp tolerance, could be identified in any of the ten analyzed animal species (numbers in bold). They are important for species recognition in mixtures. The 2bp limit of resolution of CE-TBP, already reported in a previous work (Braglia, Manca, Gianì, Hatzopoulos, & Breviario, 2017), has been further assessed and can be substantially appreciated in Fig. 2 where the peaks of beef and horse of 459 and 462 bp respectively, look very well separated.

3.2. The aTBP method: application to DNA/DNA or w/w mixtures

One of the key features of TBP as a method for genotyping is its versatility of application, meaning that the same TBP primers pair can efficiently amplify the beta-tubulin target sequences present in the genome of multiple species, with no need for prior genomic information. This is particularly useful in the analysis of mixtures since the diagnostic peaks corresponding to each of the species present can be promptly recognized, as clearly exemplified in the graph of Fig. 2 where peaks corresponding to either horse or beef can be easily recognized in a mixture made up by an equal amount of DNA of each. The level of sensitivity of similar assays, performed on binary DNA mixtures, was

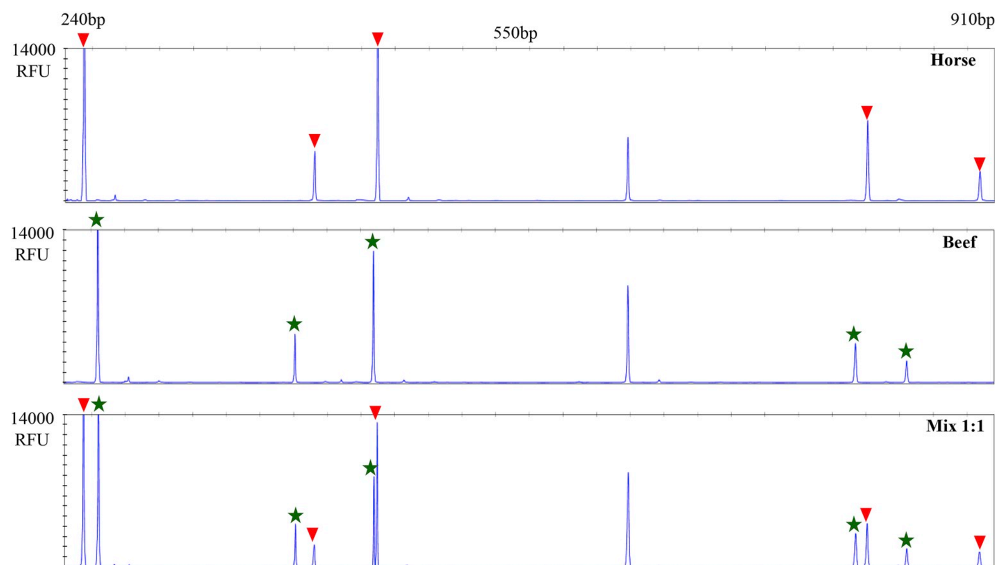
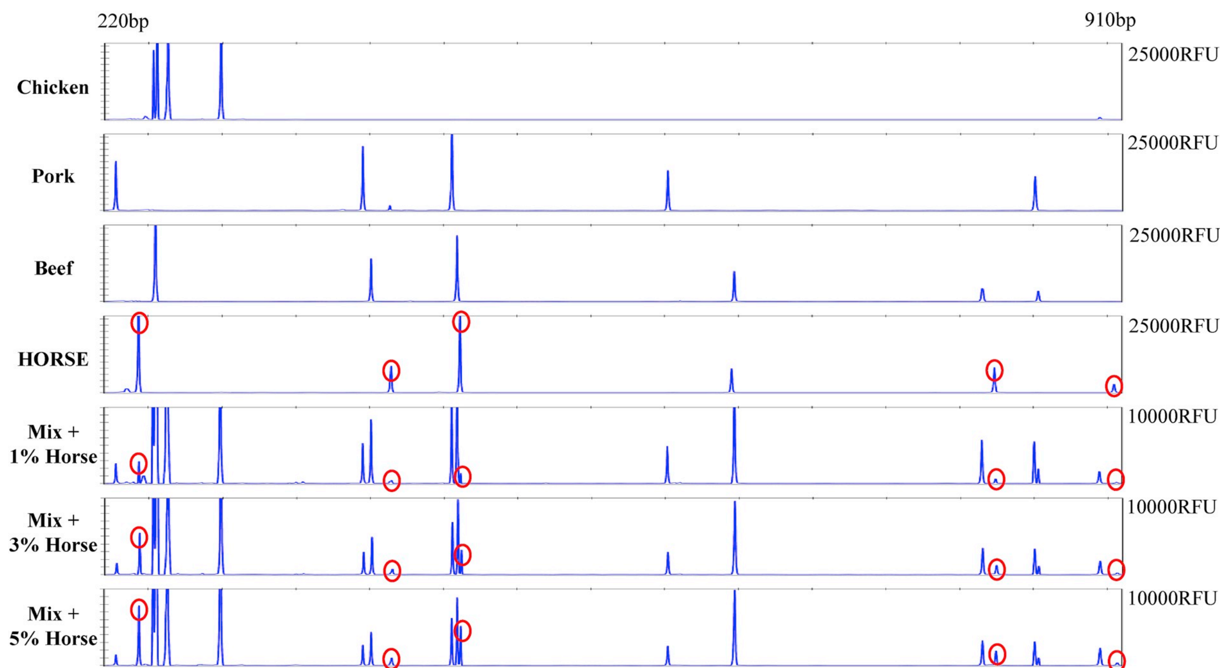


Fig. 2. CE-TBP electropherograms obtained from the aTBP analysis of a 1:1 horse and beef DNA mixture, compared with single reference profiles. Amplicons length range (from 240 to 910bp) is shown on the top and RFU values on the left side. Stars and triangles indicate horse and beef peaks respectively.

found to be around 0.5% in additional experiments where an increasing amount of either pork or horse DNA was added to chicken or beef DNA, respectively (data not shown).

A nearby 1% limit of detection could be obtained when w/w meat sample mixtures, instead of DNA's, were assayed, as shown in Fig. 3 where a mixture made up by equal amount of beef, pork and chicken (M1) was spiked with 1%, 3% and 5% increasing amount of horse meat. As shown by the circles, horse-specific peaks could already be detected at the 1% level of addition and their height gradually increased at the higher percentage values, over the same loading of amplified DNA. The increase resulting from the mean of three independent PCR amplifications, was almost linear. In fact, the ratios obtained by comparing the sum of the RFU values of each peak attributable to horse to the sum of all peaks present in the analyzed sample were 0,03 (1% horse), 0,09



	Horse	Mix	Horse/Mix
1% Horse	4959 RFU	158733 RFU	0,031
3% Horse	12730 RFU	141301 RFU	0,09
5% Horse	15768 RFU	131438 RFU	0,12

Fig. 3. Detection of horse meat in a w/w admixture containing equal amount of chicken, pork and beef. The CE-TBP electropherograms report the aTBP profile of single samples of chicken, pork, beef and horse and that of mixtures where increasing amount of horse meat were added. Horse specific peaks are encircled. The table reports the RFU values resulting from the sum of the horse-specific peaks compared to the total RFU values of the mix. RFU numbers are the average of three independent experiments.

Table 2
Effect of cooking and freezing/thawing on the detection, by aTBP, of horse meat in a mixture (M1) containing equal amount of chicken pork and beef.

Meat sample	Treatment	Amplicon Size (bp)					
		243	415	462	825	907	
Horse	Raw	19390	4284	23048	9179	3850	RFU
	Cooked	15414	4973	17499	6588	1506	
	Defrosted	20507	6137	21513	9324	4262	
M1	Cooked	0	0	0	0	0	RFU
M1- 1% horse		430	99	525	206	0	
M1- 3% horse		1038	186	1159	391	0	
M1- 5% horse		3493	659	4015	1265	0	
M1	Defrosted	0	0	0	0	0	RFU
M1- 1% horse		2920	478	1336	663	214	
M1- 3% horse		5456	761	3236	1256	298	
M1- 5% horse		7846	1041	5161	1945	436	

RFU values of non overlapping peaks are shown. RFU numbers refer to the average of 3 independent experiments. Beef amplicon 646 and horse amplicon 647 are not reported since they overlaps in mixed samples.

(3% horse) and 0,12 (5%) respectively (Table in Fig. 3). Similarly, a 1% limit of detection, was obtained in experiments where increasing 1%–5% w/w amount of pork meat was added to a triple ingredient mixture (M2) made up by a 1:1:1 ratio of beef, horse and chicken meat (data not shown). Remarkably, similar trend and limit of detection were also observed when the M1 mixture spiked with horse meat, was analyzed either after freezing/thawing or after cooking treatments (Table 2). It has been widely reported that cooking, more than other treatments, leads to DNA degradation and this may hinder the correct detection of species by standard molecular tools. However, despite the presence, in the cooked samples, of an abundant amount of degraded DNA, as ascertained by agarose gel electrophoresis (Supplementary Fig. 2), enough target sequences are left to allow TBP amplification and to reliably detect the presence of horse-specific amplicons down to the 1% spiking level, as shown in Table 2. Detection in cooked admixtures was even characterized by an appreciable linear response maintained up to the 825 bp long fragment, while only the longest diagnostic peak of 907 bp went lost. However, the same 907 bp long fragment was still readily detectable when the assay was performed on a single, cooked sample of horse meat.

3.3. The aTBP method: application to market products

The aTBP analysis for meat authentication was then applied to 25 different products sold in the market, made up by either one, two or three ingredients of animal origin, as reported in the label (Table 4). All of them have been analyzed by aTBP for their actual composition but here we restrict our description to the assays reported in Table 3. This simplified table restricts the data to single, species-specific diagnostic amplicons so that the correspondence between what was found and what declared in the label can be easily appreciated. In fact, squared numbers, indicating the presence of undeclared species could be found just in the Bolognese sauce and Lasagna samples while the composition of the other food products was in accordance to the label. The lack, in some sample such as cannelloni or lasagna, of the longer amplification fragments specific for either pork or beef, likely due to harsh heat treatments that cause a relevant degradation of the DNA, has no consequence on their detection. Apart from the two aforementioned exceptions, we found complete correspondence to the declared composition in the remaining 23 samples, ascertained by the detection of two species-specific diagnostic peaks, at the least. The overall result of our aTBP assay performed on the 25 market products is reported in Table 4.

4. Discussion

The proposition of a new method to ascertain the identity of raw meat and composition of processed meat products stems out from the conviction that there is no perfect analytical tool capable of providing an answer for all the problems that may be encountered in this field, as yet. A field, that of meat substitution and adulteration, that has a long standing record of disclosure of many different frauds and contaminations. Typicall unexpected, they could sometime involve exotic species, like kangaroo or buffalo, or species like rat, mouse, dog and cat, causing a real threat to public health, because they may transmit pathogens by escaping hygiene controls. This wide range of possibilities favors those DNA-based recognition methods that can be easily and vastly applicable, such as aTBP. Based on a single PCR reaction, the aTBP method releases, after the separation of the fragments by capillary electrophoresis, a distinct profile for any of the tested species and, in mixtures, leads to the straightforward identification of the different ingredients, declared or not in the label. Its limit of detection, close to 1%, in w/w mixtures, is generally accepted as a borderline between accidental and fraudulent contamination. In this paper we have shown that the aTBP method can assign to each of ten animal species, chosen among those most frequently consumed by humans or those that can accidentally contaminate the processed products, a specific genomic profile that is made up by a different number of peaks, each representing amplicons of different sizes. This lays the foundation for an aTBP data base that, because of its vast field of application, can be easily and widely broaden. In addition, in any of the aTBP profiles, discrete peaks that are exclusively attributable to single species can be recognized by multiple comparison. These diagnostic peaks are very useful for species recognition in admixtures, as verified in many of the 25 market products that have been analyzed. The true molecular identity of the aTBP products as beta-tubulin introns has been confirmed by multiple DNA sequencing runs and is further substantiated by the comparison between the sizes of the aTBP amplicons and those inferred *in silico* from the corresponding beta-tubulin gene sequences retrieved from the Ensembl genome browser. As anticipated, the few minor inconsistencies found can be attributed to either an imperfect resolution of the fragments by CE or to the lack of a robust confirmation of the DNA sequences deposited or to the likely presence of few allelic variants, remained unidentified as yet. When a similar approach to that of aTBP was performed on grape, an entirely new beta-tubulin allele was uncovered that was not previously annotated in the referenced *Vitis vinifera* genome sequence (Gavazzi et al., 2016). In chicken, allelic variants contribute to define the four diverse combinations found in different meat samples and food preparations. Similarly, additional polymorphic traits were observed in goat (4 peaks), turkey and sheep (2 peaks each).

aTBP can also provide some information about quantity, as we have shown on two kinds of mixtures: DNA over DNA or single species raw material over admixtures (w/w). In all cases a consistent and reliable detection around the 1% level was found. Linearity of the detection, that is an increase of the RFU values in relation to a corresponding

Table 3
Peaks detected by the aTBP method, in various representative food samples.

	Peak Size (bp)																				
Sample	229	243	253	263	271	299	301	395	401	415	456	459	462	602	816	825	851	853	896	907	
Chicken			9608	12636	4971	10765	8444												1950		RFU
Beef									13471			17727			4564			6119			
Pork	2333							3888			5814			2827			1937				
Horse		19514								5284			18460			6588				1506	
Ravioli p.n. 16	20566		11419	30829	3807	6669	770	5402	5036		30745	9998		4357	375		1120				
Beef and pork burger p.n. 17	2309							1389	2623		9056	11302		1299	2851		2442	651			
Meat sandwich p.n. 20	17394							3966	8132		28143	22605		7590	1374		2400	308			
Tortellini #1 p.n. 18	9735							7796	10775		31284	23533		8098	6305		9548	3189			
Cannelloni p.n. 23	26569							3860	451		16050	745		2129							
Bolognese Sauce p.n. 21	249	13383	3536	6456	462	6211		507	835	193	504	979	2541	155		124					
Lasagna p.n. 22	31182	1007		2221	196	258	528	4111	3078		19767	6838		1313							

Only diagnostic peaks for the four species are reported
Squared numbers indicate the presence of non declared ingredients

Table 4
Detected composition, through aTBP analisys, of the meat-based-ingredients present in 25 different products sold in the market.

	Food product	Declared meat ingredients	Detected Composition										
			Tu	Ch	Be	Po	Ra	Ho	Go	Sh	Mo	Hu	CO
1	Bresaola	Beef			▒								✓
2	Minced meat	Beef			▒								✓
3	Stock cube	Beef			▒								✓
4	Beef burger	Beef			▒								✓
5	Cordon Bleu	Chicken, Turkey	▒	▒									✓
6	Kebab #1	Chicken, Turkey	▒	▒									✓
7	Chicken burger	Chicken		▒									✓
8	Kebab #2	Chicken		▒									✓
9	Canned chicken	Chicken		▒									✓
10	Rabbit burger	Rabbit					▒						✓
11	Wurstel	Chicken, Turkey, Pork	▒	▒		▒							✓
12	Turkey salami	Turkey 85%, Pork	▒			▒							✓
13	Original wurstel	Pork				▒							✓
14	Salami	Pork				▒							✓
15	Raw ham	Pork				▒							✓
16	Ravioli	Pork, Beef, Chicken		▒	▒	▒							✓
17	Beef and pork burger	Beef 34%, Pork 51%			▒	▒							✓
18	Tortellini #1	Beef 47,5%, Pork 47,5%			▒	▒							✓
19	Tortellini #2	Beef 12%, Pork 51,6%			▒	▒							✓
20	Meat sandwich	Beef 50%, Pork 50%			▒	▒							✓
21	Bolognese Sauce	Pork 9,5%, Beef 16,5%		█	▒	▒		█					X
22	Lasagna	Pork 24%, Beef 17%		█	▒	▒							X
23	Cannelloni	Pork, Beef			▒	▒							✓
24	Goat salami	Goat 75%, Pork 25%				▒			▒				✓
25	Beef salami	Beef			▒								✓

Tu: Turkey, Ch: Chicken, Be: Beef, Po: Pork, Ra: Rabbit, Ho: Horse, Go: Goat, Sh: Sheep, Mo: Mouse, Hu: human, CO: Compliant with declared.
On the label of some products are indicated the percentages of the meat-based-ingredients shown in the table
Light gray indicates the correspondence between what is declared on the label and what was found with the aTBP analysis.
Dark gray indicates the presence of undeclared species

increase of the contaminating species, has also been documented. Clearly, aTBP cannot provide a rigorous quantitative determination, task achievable with RT-PCR or ddPCR, both using specific probes, but it supports a most valuable combination of an immediately appreciable qualitative profile with a trustworthy estimate of the amount. After all, the 1% limit of detection, more commonly applied to DNA/DNA than to w/w measurements, relates to a most pragmatic, widely adopted approach, defining an appropriate level at which trace contamination can be distinguished from deliberate substitution. It is also true that if fraud of economical relevance is the driving purpose, it is not really necessary to detect additions of undeclared meats at a level below 1%, which may instead become critical for allergenic reactions. This is somewhat proven by the two market products, Bolognese sauce and the meat filling of lasagna, where an abundant amount of undeclared, economically more convenient chicken meat was found in addition to beef and pork. Somewhat surprising was also the finding that an aTBP genomic profile could be obtained from raw material and admixtures after cooking in a microwave oven in the presence of degraded DNA, although this may not be the case for canned products where DNA sizes are typically lower than 300bp. Even so, our finding is of importance because it has been reported that cooked meat products are found more adulterated than raw meat. Therefore, the effect of high temperature on

nuclear DNA degradation, referred to be relevant in cooked meat (Aslan, Hamill, Sweeney, Reardon, & Mullen, 2009), could sometime be overestimated and the failure of detecting specific diagnostic fragments attributed to other factors. Identity of the animal species could be one of the key factors since some species could be more easily recognizable than others, depending on the experimental conditions, as also recently shown for minibarcoding. Developed to overcome the limits of the Sanger assisted full barcoding, when analyzing mixtures, turned out to be biased in favor of some species with respect to others (Hellberg, Hernandez, & Hernandez, 2017). While successfully applied to the search of turkey or duck in different processed products, it performed inefficiently in the detection of chicken and beef (Hellberg et al., 2017). In more general terms, food sequencing/metabarcoding implementation currently requires elaborate knowledge of genomes and a relevant bioinformatic support (Ripp et al., 2014) and is still dependent on very laborious protocols for the preparation (Kappel, Haase, Kappel, Sotelo, & Schroder, 2017) requiring several steps as DNA digestion, adaptor ligation, clonal amplification, each followed by purification steps, estimation of the DNA amount and quality check by CE and others. Therefore, metabarcoding is not yet straightforward and convenient as it sounds, because of the high costs of the equipment and maintenance and a reported success rate in DNA sequencing that runs around 68%–80% for full barcoding approaches down to 38.3% for minibarcoding, in highly processed food products (Hellberg et al., 2017). Typically, deep DNA sequencing works well, that is that leads to an easy recognition of the species, if present as a singleton, whereas metabarcoding of mixed products, requiring the assignment of multimapped reads, shared among genomes of different species, is more challenging and requires more refined and careful data elaboration. For all these reasons aTBP may represent a useful, credible, simpler, more affordable, alternative and highly versatile tool for food profiling. Similar to DNA sequencing-based methods, the results of the aTBP method can be used to inform the design of species-specific probes and/or to generate digital genomic labels.

5. Conclusions

With this contribution, we aim to propose the integration of aTBP in the selected list of markers useful for animal food authentication. Relying on widespread nuclear target sequences, aTBP provides a remarkable combination of a rapid DNA profiling, applicable to raw materials, mixtures and products, with a useful quantitative estimation. Future applications to additional animal species, including fish and processed products, will further verify the specificity, reliability and robustness of the aTBP method.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2019.107010.

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